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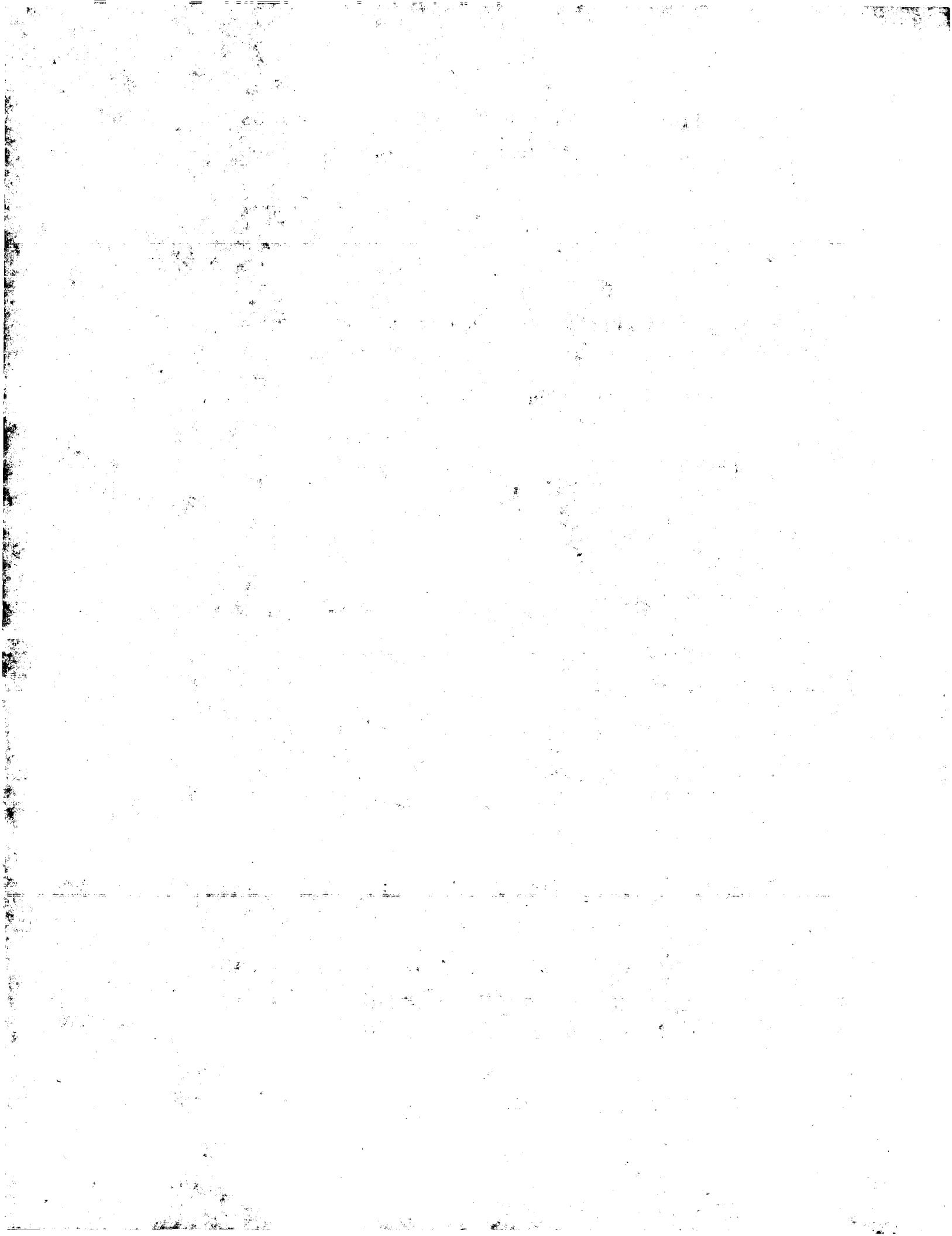
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Cornelis et al.

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Filed: July 25, 2001

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SITE, VECTOR CONTAINING SAME AND
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Enclosed is a copy of Priority Document 99200216.2 EP filed January 26, 1999 for the above referenced application.

Respectfully submitted,

Allen C. Turner
Registration No. 33,041
Attorney for Applicant(s)
TRASKBRITT
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

Date: April 16, 2004

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

99200216.2

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office
Le Président de l'Office européen des brevets
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**Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation**

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Internal ribosome entry site (IRES), vector containing same and the uses thereof

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OAV1 / RUB /IRES / 001-036 / EP**Novel internal ribosome entry site, vector containing same and the uses thereof**Field of the invention

The present invention relates to an isolated and/or recombinant nucleic acid molecule, preferably a cloned DNA sequence element, that can be incorporated into expression vectors for improving translation of a given mRNA and to enable said translation of the mRNA in a cap-dependent but also, more importantly, in a cap-independent manner in eukaryotic cells. The DNA sequence element comprises the so-called internal ribosomal entry site, abbreviated as and called hereafter IRES.

Summary of the invention

It has been found by the current invention that two isoforms, p110 and p58 of PITSLRE protein kinase, can be translated from the same p110 ($\alpha 2-2$) mRNA by an internal ribosome entry process. This means that p110 and p58, two proteins with putative different functions, are translated from a single mRNA species by using two AUGs within the same reading frame. These two proteins share the 439 C-terminal amino acids that contain the kinase domain. The IRES in the polycistronic p110 mRNA is the first IRES completely localized in the coding region of a cellular mRNA. Moreover, it was surprisingly found that the IRES element is cell cycle regulated. Translation of p58 occurs in the G2 stage of the cell cycle.

Background of the invention

The PITSLRE protein kinase family is a large supergene family related to the master mitotic protein kinase, p34cdc2. The members of the PITSLRE kinase family are encoded by three tandemly duplicated and linked genes: α , β and γ . The genes are located on human chromosome 1p36.3. Two of the three genes are almost complete duplications and express nearly identical products. According to Xiang et

al., 1994 a complex pattern of alternative mRNA splicing and promotor utilization give rise to different translation products: $\alpha 1$ (p65), $\alpha 2-1$ (p110), $\alpha 2-2$ (p110), $\alpha 2-3$ (p110), $\alpha 2-4$ (p90), $\beta 1$ (p58), $\beta 2-1$ (p110), $\beta 2-2$ (p110). All isoforms contain all of the $\beta 1$ ORF, situated at the C-terminal end. Divergence between the isoforms is localized to the amino-terminal region.

At present the function of PITSLRE kinases remains elusive. The reason for the high number of different isoforms is also not clear. Only the p110 and p90 isoforms contain a 30-amino acid region comprised primarily of glutamic acid (83 %) (Xiang et al., 1994). Shorter glutamic acid sequences can be found in all isoforms immediately following the first translational start site for the $\beta 1$ isoform. Several highly basic regions, which could function as bipartite nuclear localization sequences, are found only in the p110 isoforms (Xiang et al., 1994).

The yeast two-hybrid system revealed a direct interaction between the RNA-binding protein, hRNPS1, and the p110 isoforms but not with the smaller isoforms (Loyer et al., 1998).

The wide variation in the expression patterns of the different isoforms and their levels of expression points to isoform specific functions. While p110 isoforms are ubiquitously expressed in asynchronous cell populations, overexpression of p58 in eukaryotic cells leads to a late mitotic delay due to an apparent failure of cytokinesis. The cells are sequestered at late telophase for an extended period of time (Bunnell et al., 1990). The rate of cell growth in these cells is greatly diminished. Conversely, diminished p58 mRNA levels in CHO fibroblasts are associated with enhanced cell growth, as measured by increased rates of DNA replication (Meyerson et al., 1992). These observations suggest that p58 might participate in normal regulation of the cell cycle or cell death. Additionally, the chromosome region 1p36.3 is often deleted in neuroblastoma and many other tumors. Deletion of this chromosome region occurs late in oncogenesis and is correlated with aggressive tumor growth, suggesting that one or more tumor suppressor genes may reside here (Eipers et al., 1991). Another observation pointing to a role for PITSLRE kinases during cell cycle progression is that during early

embryogenesis in *Drosophila*, when exponential proliferation takes place, the expression level of PITSLRE specific transcripts, encoding the p110 homologue, is maximal. At a later stage when cell proliferation is attenuated, PITSLRE expression drops dramatically (Sauer et al. 1996).

Polycistronic messengers as such occur frequently in prokaryotic systems. It had been a long-established misunderstanding that said polycistronic messengers did not occur in eukaryotic systems because of the presence of the so-called "cap" at the start of mRNA; said cap is necessary for a correct initiation of translation. However initiation of translation of the majority of eukaryotic cellular and viral mRNAs results from attachment of ribosomes to the m⁷ G cap at the 5'-end of the mRNA followed by linear scanning to the initiation codon. Initiation of translation of a smaller number of eukaryotic mRNAs is 5'-end- and cap-independent, and instead results from direct attachment of ribosomes to an internal ribosomal entry site (IRES) within the 5' non-translated region (5' NTR) of the mRNA.

IRES elements were first reported in picornaviral mRNAs which are naturally uncapped but nonetheless efficiently translated (Jang et al., 1988, J. Virol., 62:2636-43). Generally IRES cannot be identified by sequence homology; known IRES have been identified and defined functionally (Mountford and Smith, 1995, TIG, 11(5):179-184). It appears that the conformation of the IRES sequence enables the binding on the ribosome.

It would be useful to have identified a sequence element that endows any desired gene with the ability to be efficiently translated and to be translated in a cap-independent manner in particular. In addition it would be of great advantage to isolate a sequence element which is cell cycle regulated. So it would be extremely useful to have IRES sequence elements with a preferably high translational efficiency to use in expression vectors to control mRNA translation and therefore protein synthesis, as well as to use in gene therapy vectors.

Detailed description of the invention

The current invention relates among others to the feature that p58 is produced from the PITSLRE p110 ($\alpha 2$ -2) mRNA by a mechanism of internal initiation of translation during the G2 stage of the cell cycle. An embodiment of this invention is disclosed hereafter.

An SV40 early promoter driven p110 PITSLRE isoform ($\alpha 2$ -2) fused to an E-tag at its C-terminal end was constructed. This plasmid was stably transfected in the IL-3 dependent pro B-cell line Ba/F3. This system has the advantage that cells can be synchronized in the G1 stage by growth factor depletion. Subsequent stimulation with IL-3 drives the cells simultaneously through further stages of the cell cycle. Via immunoblotting using anti E-tag the expression pattern of the transfected PITSLRE construct was studied during cell cycle progression in the Ba/F3-p110PITSLRE transfectants. The p110 isoform was constitutively present during the different stages of the cell cycle. However, only during the G2/M stage a 58 kDa form was co-expressed (figure 1a). Cell cycle progression was followed by FACS analysis (figure 1b). To make sure that this expression pattern was not a clone dependent phenomenon several other clones were analysed. All displayed a similar pattern.

Also a minor band of about 100 to 105 kDa was detected. It has been described previously that the p110 isoforms have two potential translational starts near the start of the 5'NTR region. They are separated by 174 bp and they are located in two adjacent exons. A possible mechanism however has not been suggested. Maybe this alternative initiation of translation is a consequence of leaky ribosome scanning. The alternative AUG (at position 283) is located in a more favourable region for initiation of translation compared to the first AUG (position 112)(table 1).

The origin of the p58 product was initially thought to be the result of a proteolytical process. It has been suggested that glutamic acid regions present in the p110 isoform are potential points of specific proteolytical cleavage. Recently, a possible role for a caspase processed PITSLRE isoform has been suggested by studies of Fas- and TNF-induced cell death. (Lahti et al., 1995, Beyaert et al., 1997, Tang et al., 1998).

Transfectants were incubated with the broad spectrum inhibitor of caspases, zVAD.fmk and the PITSLRE expression pattern was analysed. As figure 1a shows, zVAD.fmk did not affect G2 specific expression of p58, excluding the possibility that p58 was generated by proteolytic processing of the p110 isoform by resident caspases (fig. 2A)

The p110 isoform contains at its C-terminal end the ORF of isoform β 1. Therefore the G2-specific p58 protein was probably translated using the internal AUG as an initiation codon. To check this the internal methionine was mutated to alanine and the mutant cDNA was transfected into the Ba/F3 cell line. Interestingly this mutation completely knocked out the p58 expression in the G2 stage of the cell cycle whereas p110 expression remained unchanged (fig. 2A, 2B).

The expression of cyclin B1 as a marker for the G2 stage of the cell cycle was used as control. Cyclin B1 is synthesized during late S, maximally expressed during the G2 and finally degraded during the anaphase.

Since expression of the transfected gene was controlled by a constitutive early SV40 promoter, regulation of p58 expression at the transcriptional level was excluded.

Next, it was determined whether p58 and p110 were both derived from one mRNA. It is possible that p58 is translated from another, second, messenger derived from the initially transfected cDNA. This second messenger could be produced by cleavage of the transfected messenger by a specific ribonuclease or could be induced by a cryptic promoter element present in the upstream sequence. The exogenous mRNA pattern was followed during cell cycle progression by Northern blot analysis with an E-tag specific probe in the Ba/F3 transfectants (figure 2A). One single messenger of 3.4 kb in all stages of the cell cycle was found at a constant expression level. The 3.4 kb band was absent in non transfected cells. No additional messenger in the G2-specific cell lysates was detected. This shows that the p110 (α 2-2) mRNA, encoding p110 PITSLRE (α 2-2), gives rise to a second PITSLRE protein kinase of 58 kDa.

Endogenous PITSLRE protein kinase expression during cell cycle progression was also examined. As shown in figure 3, analysis of the Ba/F3 parental cells during cell

cycle progression revealed a similar pattern compared to the Ba/F3/p110PITSRE transfectants. p110 isoforms are permanent present during the different stages of the cell cycle. In accordance with the observations in the transfectants, a strongly enhanced expression of p58 was detected in the G2-stage.

The invention thus relates to an isolated and/or recombinant nucleic acid molecule, preferably DNA, encoding at least a functional part of an eukaryotic internal ribosomal entry site, which site in the mitotic PITSRE protein kinase gene comprises the sequence as depicted in SEQ.ID.NO. 1 or a functional part thereof.

In a broader aspect the invention thus concerns a nucleotide sequence enabling a cell cycle dependent initiation of translation of mRNA, wherein said sequence is an IRES sequence and more preferably wherein the cell cycle dependency is a G2 cell cycle dependency.

To the present invention also belongs a nucleic acid molecule comprising at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence at least substantially homologous thereto.

In another embodiment the nucleic acid molecule according to the invention relates to at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence which hybridizes under conventional conditions to at least a part of said sequence or its complementary sequence.

Unexpectedly it has been found that SEQ.ID.NO.4, being an overlapping sequence of SEQ.ID.NO.5 and SEQ.ID.NO.1, has corresponding IRES functionality according to the current invention. Therefore part of the invention is an isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 4, but also an isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 5 and in addition thereto an isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 6.



To the scope of the invention also belongs a chimeric gene comprising the following operably linked polynucleotides:

- a) a nucleic acid molecule according to the invention and
- b) one or more control sequences.

Part of the present invention is also a vector comprising at least said nucleic acid molecule or comprising said chimeric gene above mentioned whereas an eukaryotic host cell may comprise said nucleic acid molecule or may comprise the chimeric gene according to the invention.

Said vector can be conveniently an expression vector containing at least a single promoter. A derived expression system comprising an eukaryotic host cell according to the invention forms part of the invention as well.

Another aspect of the invention is a method for cap-independent translation of mRNA by including in an expression vector, a translation control element or analogues thereof having the nucleic acid molecule as set forth in SEQ.ID.NO.1 and/or SEQ.ID.NO.4.

The sequences of the invention can be used to induce a cell cycle dependent initiation of translation in eukaryotic cells. In particular said sequences can be used wherein the sequence is a cell cycle dependent IRES sequence, more preferably a G2-dependent IRES sequence.

In addition said vector or any of the sequences according to the invention can be used for the preparation of a pharmaceutical composition for the treatment and/or prevention of a disease by gene therapy.

In order to clarify what is meant in this description by some terms a further explanation is hereunder given.

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the

molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog.

"Recombinant nucleic acid molecule" as used herein refers to a polynucleotide of genomic, cDNA, semisynthetic or synthetic origin which, by virtue of its origin or manipulation (1) is linked to a polynucleotide other than that to which it is linked in nature or, (2) does not occur in nature.

An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors.

A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

"Control sequence" refers to regulatory DNA sequences which are necessary to affect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a

minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is used.

The terms "protein" and "polypeptide" used in this application are interchangeable. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus peptides and oligopeptides are included within the definition of polypeptide. This term does also refer to or include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

"Fragment of a sequence" or "part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity of the original sequence referred to, while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid

sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a maximum of about 50 amino acids in length, preferably a maximum of about 30 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

"Transformation" as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer. The polynucleotide may be transiently or stably introduced into the host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. Many types of vectors can be used to transform cells. These transformation methods are known to persons skilled in the art.

"Functional part of" means that said part to which subject it relates has substantially the same activity as the subject itself, although the form, length or structure may vary.

The term "substantially homologous" refers to a subject, for instance a nucleic acid, which is at least 50% identical in sequence to the reference when the entire ORF (open reading frame) is compared, where the sequence identity is preferably at least 70%, more preferably at least 80%, still more preferably at least 85%, especially more than about 90%, most preferably 95% or greater, particularly 98% or greater. Thus, for example, a new nucleic acid isolate which is 80% identical to the reference is considered to be substantially homologous to the reference.

Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridisation experiment under, for instance, conventional or preferably stringent conditions as defined for that particular system.

Similarly, in a particular embodiment, two amino acid sequences, when properly aligned in a manner known to a skilled person, are "substantially homologous" when more than 40% of the amino acids are identical or similar, or when more preferably more than about 60 % and most preferably more than 69% of the amino acids are identical or similar (functionally identical).

"Sense strand" refers to the strand of a double-stranded DNA molecule that is homologous to a mRNA transcript thereof. The "anti-sense strand" contains an inverted sequence which is complementary to that of the "sense strand".

"Cell cycle" or "cell division" means the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. The cycle is divided into periods called: G₀, Gap₁ (G₁), DNA synthesis (S), Gap₂ (G₂), and mitosis (M).

"Expression" means the production of a protein or nucleotide sequence in the cell itself or in a cell-free system. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications.

The present invention also relates to nucleic acid molecules hybridizing with the above-described nucleic acid molecules and differ in one or more positions in comparison with these as long as they encode a comparable protein. By "hybridizing" it is meant that such nucleic acid molecules hybridize under conventional hybridization conditions, preferably under stringent conditions such as described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). An example of one such stringent hybridization condition

is hybridization at 4XSSC at 65 °C, followed by a washing in 0.1XSSC at 65 °C for one hour. Alternatively, an exemplary stringent hybridization condition is in 50 % formamide, 4XSSC at 42 °C. The invention also relates to nucleic acid molecules the sequence of which differs from the nucleotide sequence of any of the above-described nucleic acid molecules due to the degeneracy of the genetic code.

Homology further means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of said nucleic acid molecules are, for example, variations of said nucleic acid molecules which represent modifications having the same biological function, in particular encoding proteins with the same or substantially the same biological function. They may be naturally occurring variations, such as sequences from other varieties or species, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques. The allelic variations may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a nucleic acid molecule according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

The current invention is further described and explained by way of the following non-limiting examples. A section disclosing the materials and methods used in the Examples is included too.

EXAMPLES

Example 1

The internal initiation of translation on the PITSLRE p110 (α 2-2) mRNA is mediated by an IRES element present in the coding region.

A possible mechanism accounting for the synthesis of p58 is leaky ribosome scanning (Kozak, 1989, 1991). According to this model proposed by Kozak and coworkers, the small subunit of the ribosome first recognizes the 5' terminal cap structure of an mRNA and then scans the mRNA sequence in a 5' to 3' direction for potential AUG initiation codons. Often, but not always, the first AUG is utilized. Whether this AUG is selected or ignored depends largely on the sequence context surrounding it. An optimal nine nucleotide consensus sequence, 5'-CC(A/G)CCAUGG-3', has been derived on the basis of extensive mutagenesis experiments (Kozak, 1986). The presence of a purine in position -3 is most important for efficient AUG usage. In the absence of a -3 purine, the presence of a guanosine at position +4 is essential. Ribosomal subunits that fail to initiate at the first AUG can continue their search for an AUG in a more favorable sequence context.

Inspection of the PITSLRE cDNA sequence reveals a poor to moderate match around the first AUG and a very poor match for the nucleotides flanking the AUG of p58 (table 1). If the scanning mechanism would be used for translational initiation of p58, the ribosomal 43S ternary complex would bind at the 5' end of the mRNA and would scan 1011 nucleotides, bypassing seventeen AUG codons to initiate protein synthesis at the eighteenth AUG codon. Several of the upstream AUG codons are in a more favourable context to initiate protein synthesis than the eighteenth AUG (table 1). Because the observations according to the current

invention are not compatible with the leaky scanning model, the possibility of the presence of an IRES sequence in the coding region of the PITSLRE mRNA was examined. Bicistronic mRNAs have been effectively used *in vivo* to demonstrate the existence of IRES sequences in both viral and cellular mRNAs. A plasmid vector in which the SV40 promotor drives the transcription of a capped bicystronic transcript was constructed in the present invention (fig. 4A). The first cytron, encoding luciferase (Luc) should be translated by the conventional cap-dependent scanning mechanism. However, as ribosomes fail to continue scanning through the intercistronic spacer (ICS) insert, the second cytron, encoding β -galactosidase (LacZ), should be translated only if the preceding sequence contains an IRES.

The region starting from position 121 was first subcloned and ending up at the internal initiation codon ATG(p58) (position 1126) by PCR amplification (di-1, figure 4 B) and the plasmid was transient transfected into the 293 T cell line. The translation products were monitored in enzymatic assays (fig. 4 C) and by western blotting (figure 5A). As expected, the dicystronic mRNA produced luciferase. Interestingly, the same lysate was also positive in the LacZ activity test. Western blot analysis showed that both translation products were of the correct size excluding the occurrence of fusion proteins (figure 5). The observation that LacZ is translated from the dicystronic transcript suggests the presence of an internal ribosomal entry site in the ICS.

To exclude the possibility that the function of the potential IRES-element is to promote the transfer of initiation-competent ribosomes from the termination codon of the upstream cistron to the initiation codon of the downstream cistron, a hairpin near the 5' end was inserted (hairpin di-1). Figure 5B shows that this modification only negatively influences translation of the first cistron while LacZ expression remains unaffected. If enhanced ribosomal readthrough is responsible for the ICS responsible stimulation of LacZ then this activity should be reduced by an equivalent amount.

In addition two constructs were made in which the p58 coding region in p110 PITSLRE in frame was exchanged by the coding region of luciferase. In one of

them a frame shift was induced in the PITSLRE specific region by deleting two nucleotides (926/927). Both cDNAs were transiently transfected into 293T cells. The respective translation products of the in frame fusion mRNA and the frame shift fusion mRNA were analysed by immunoblotting with anti-Luc antibody. In accordance with p110 PITSLRE, in the cell lysates of the in frame fusion mRNA two translation products were detected: p110-Luc (130 kDa) and Luc. In the cell lysates of the frame shift fusion mRNA only Luc was detected. The frame shift abrogated translation of the fusion protein, while internal translation of luciferase conferred by the PITSLRE IRES element remained unaffected.

To exclude the possibility that LacZ was expressed from a monocistronic mRNA that might have been generated if the IRES element had sites for cleavage by a specific ribonuclease or if the IRES had a cryptic promotor element Northern blot analysis was performed. The same mRNA was detected both by luciferase and LacZ probes. This observation prooved that both cistrons were translated from an intact dicystronic form of the mRNA.

To determine whether the cellular environment in the G2 stage of the cell cycle is more supportive for internal initiation of translation mediated by the PITSLRE IRES element the dicystronic vector was stably transfected into the Ba/F3 cell line. Several clones were obtained and analysed during cell cycle progression. Luciferase and LacZ activity were determined in cell lysates prepared at different time points during cell cycle progression. As shown in figure 6, the G2 stage is associated with enhanced LacZ activity, corrected for the amount of mRNA by measurement of luciferase activity. A 3-5 times enhanced LacZ/Luc ratio was measured in the G2 stage compared to the G1. Hence, it seems that the G2-stage facilitates the internal initiation.

The fact that LacZ activity can still be detected at the G1 stage of the cell cycle might be a consequence of the higher stability of the protein compared to the stability of p58.

Table 1

Nº AUG	AUG position	AUG (CC(A/G)CCAUGG context
1	112 (p110)	CUCAAAU GG
2	119	GGG UGAUGA
3	152	UUU AGAUGA
4	227	UUC UGAUGA
5	283* (p105)	ACUG CAUGG
6	328*	ACUC UAUGG
7	350	AGAAG AGAUGA
8	382*	AGCAAA UGU
9	416	AAAAG AGAUGA
10	440	AAAG CAUGC
11	519	CGGG AAUGG
12	544*	GGGAAA U ^G GG
13	578	GGGG AAUGA
14	581	GAA UGAUGG
15	646*	GCAAG AGUGC
16	757*	GAAC GAUGA
17	874*	AGAAA U ^G GG
18	1126* (p58)	AAGAAA UGA

*: in frame AUG

in bold : matches with consensus sequence according to Kozak, 1986.

Example 2

Characterization of the PITSLRE IRES element

The cloned fragment in the ICS in the dicystronic vector, di-1, contains 1000 nucleotides. Regions described to contain IRES activity are maximal 450 bp long. To map the region with IRES activity in the p110 mRNA a series of dicystronic plasmids was generated containing decreasing lengths of sequence coding for p110PITSLRE. Different fragments (figures 4 and 5) were inserted into the intercystronic spacer region (di 2 to di 6) and transient transfected the dicystronic plasmids into the 293 cell line. As expected, all dicystronic mRNAs produced luciferase. The luciferase activity was used as an internal control for the different transfection efficiencies of the plasmids.

The ability of the truncated sequences to promote internal ribosomal entry on the dicystronic mRNA was compared to di-1.

The constructs containing fragments of the 5' end of the analysed region did not score in the LacZ activity test (figure 4, di-2, di-3). Extended deletions at the 3' end completely abrogated internal initiation, suggesting that the IRES element is situated upstream of the internal initiation codon.

Interestingly, deletion of 347 nts at the 5' end had no effect on the activity of the downstream cystron (di-4). However larger deletions of 624 nts (di-5) and 786 nts (di-6) resulted in a corresponding reduction of internal ribosome entry of 25% and 50%, respectively. This partial loss of IRES activity may reflect loss of secondary or tertiary structure elements that confer positive or negative effects or loss of protein-binding sites. The IRES activity harbored in the ICS of di-4 is comparable in strength with the activity in the ICS of di-1. The fact that it is possible to make a small deletion at the 3' end without a dramatic loss of activity has also been described for the *c-myc* IRES (Stoneley et al., 1998).

Analysis of the translation products from the different dicystronic mRNAs by Western blot analysis, shown in figure 5A, shows that di-1, di-5 and di-6 express LacZ of the expected size. Di-4, which also scored positive in the LacZ activity test,

expresses a larger LacZ specific translation product. It seems that deletion of a small region immediately upstream of the AUG of internal initiation of translation does not interfere activity, in casu ribosome binding, but affects the site of initiation of translation taken by the ribosomes. In various viral IRESes 3'end deletions located within the IRES complete ablate ribosomal entry.

Example 3

Structural features of the PITSLRE IRES element

The importance of RNA secondary and tertiary structure for IRES function emerges from a comparison of the sequences and secondary structures of different IRESes. A common RNA structural motif involved in the internal initiation of cellular mRNAs has been proposed by Le and coworkers (Le and Maizel, 1997). A common RNA structural motif, including a Y-type stem-loop followed by a stem-loop is a conserved property found in cellular IRES elements. One remarkable property of the stem-loop is that this structure is situated just upstream from the authentic initiator (Le and Maizel, 1997).

Figure 6 shows the secondary structure of a fragment of 491 bp (nt 637 to 1128) of the p110PITSLRE mRNA shown to contain IRES activity. This secondary structure is predicted by the Zuker procedure (computer programm: mfold).

Two structural domains seem to correlate with IRES activity. A Y-type stem-loop (689-823) and stem-loop (1069-1105). $\Delta G = -128.2$ kcal/mol.

MATERIAL AND METHODS

Plasmid constructions

The p110 PITSLRE cDNA was obtained by reverse transcription and polymerase chain reaction (PCR) amplification of total mRNA from human

HL-60 cells using Superscript reverse transcriptase (Life Technologies, Inc.) and High Fidelity DNA polymerase (Boehringer). The 5'- and 3'-primers used for this amplification, 5'-TGCTCTAGAGGAATTGAGCTGACGATACTTTGGCGC-3' and 5'-TGCTCTAGACCAAGCTTCACGTCCATCAAGCCGACCTCAGAA-3', respectively, gave a cDNA fragment of 2400 bp. The PCR fragment was digested with the restriction enzym *EcoRI* and cloned into the pMA58 plasmid.

This construction was used to perform site directed mutagenesis by a chloramphenicol-selection procedure using a commercially available kit (Transformer, Clontech). Briefly, this method involves simultaneously annealing of two oligonucleotide primers to one strand of the denatured double-stranded plasmid (pMA-p110PITSRE). One primer introduces the desired mutation. The second primer induces a gain of function mutation in the gene encoding chloramphenicol resistance for the purpose of selection.

To fuse an E-tag at the 3'-end of p110 PITSLRE cDNA an in frame *NotI* restriction site was introduced at the stop codon (5'-AGCCTCAAGTTCGCGGCCGCAGAGTGGACC-3'). As an *EcoRI/NotI* fragment the p110 PITSLRE cDNA was inserted in the *EcoRI/NotI* opened pSV-Sport-Etag plasmid. The latter was obtained by insertion of the E-tag as a *NotI/XbaI* fragment in the *NotI/XbaI* opened pSV-Sport plasmid.

The following primers were used for the mutation of the internal initiation codon and for the induction of the frame shift respectively: 5'-GAGGAAGAAGCGAGTGAAGAT-3' and 5'-GACAGCGAGAAAGACCAGCTCG-3'.

The dicystronic vectors were made by first cloning the PCR-fragments and LacZ gene into the puc19 plasmid performed by a three points ligation. In a subsequent three points ligation the PCR-fragment fused to LacZ gene was inserted together with the firefly luciferase gene into the pSV-Sport plasmid.

The 5'end - and 3'-end primers used for amplification of the different fragments cloned into the inter cystronic spacers of the different dicystronic vectors: di-1: sense: 5'CTAGTCTAGAAAAGTGAAAACTTAGATGAAATTG-3'; antisense: 5'

di-2: sense: idem di-1; antisense: 5' TGCATGCCATGGATGTCGTTCCGACGTTCGTGC3'.

di-3: sense: idem di-1; antisense: 5' TGCATGCCATGGCCTCTCTCATCGTCGGTGATG3'.

di-4: sense: 5' GCACGAACGTCGGAAACGACATCTAGACTAG3'; antisense: 5' CATGCCATGGCTTCCTCTCGCTGTCGCTGATGTC3'.

di5: sense:

5' CTAGCTAGACATCACCGAACGATGAGAGAGG3'; antisense: idem di-1. di6: sense: 5'- GACATCAGCGACAGCGAGAGGAAGACCAGCTAGACTAG-3'

The subsequent PCR fragments were digested with the restriction enzymes: *Xba*I and *Nco*I. These fragments were cloned together with LacZ gene as a *Nco*I/ *Sal*I fragment (from pIRES-lacZ) in a *Xba*I/*Sal*I opened Puc19 plasmid. Subsequently, the complete insert was clones as an *Xba*I/*Sal*I fragement in the *Kpn*I/*Sal*I opened pSV-Sport plasmid together with the firefly luciferase gene which was cloned as an *Kpn*I/*Xba*I fragment (from the pGL3-basic vector (Clonetech).

A stable hairpin ($\Delta G = -40$ kcal/mol) was created by introduction of a double-stranded oligonucleotide (5' CGCGTGGCGAGATTTCAGGAGTCAC3' and 5' TCGAGTGACTCCTGAAAATCTGCCA3') between the *Mlu*I and *Xho*I sites of vector pGL3-basic upstream of the luciferase gene. This was accomplished by ligation of the double-stranded oligonucleotide (with *Mlu*I and *Xho*I ends) with the *Mlu*I/*Xho*I-opened pGL3-basic vector.

Cells and DNA transfection

Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated FCS.

The IL-3-dependent mouse pro-B cell line Ba/F3 (Palacios and Steinmetz, 1985) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated FCS and 20% (v/v) conditioned medium from the WEHI-3B cells as a source of mIL-3.

293T cells were transiently transfected by a calcium phosphate precipitation method (O'Makoney and Adams, 1994). Cells were incubated for at least 4 h with the transfection solution followed by adding fresh medium. Cells were collected by centrifugation at 48 post-transfection and were further analysed.

Ba/F3 cells were stably transfected by electroporation. Before transfection, cells were collected and resuspended at 1x10⁷ cells per ml in medium. 20 µg of p110PITSRE plasmid and 5 µg carrying a puromycin resistance gene (pBSpacdeltap) (De la Luna, S. et al., 1988) was added to 0.8 ml of cell suspension. Electroporation was performed using the Easy Ject apparatus (Eurogentec) at 1500 µF and 300 V. Subsequently, the cells were resuspended in WEHI-3B supernatant supplemented growth medium. Selection was initiated 48 h after transfection in medium containing 1 mg/ml puromycin (Sigma). After one week, surviving cells were cloned by limiting dilution. Positive transfectants were selected on the basis of immunoblotting or reporter gene expression measured by enzymatic assays.

Western blot analysis

For Western blot analysis cells were lysed in a 1% NP40 lyse buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10 % glycerol, 1 mM Pefablock (Merck), 200 µ/ml aprotinin, 10 mM EDTA, 10 µg/ml leupeptin).

Total proteins were quantified in the precleared cell lysates by the Biorad assay (A295) and 50 µg of proteins were subjected to SDS-PAGE and transferred by electroblotting onto an nitrocellulose membrane. E-tag fused proteins were immunodetected with the mouse monoclonal anti-Etag antibody (1/1000 dilution) (Pharmacia). LacZ and firefly luciferase were immunodetected with the mouse monoclonal anti LacZ (1/1000 dilution) (Boehringer) and rabbit polyclonal anti-luciferase antibody (1/2000 dilution) (Promega), respectively. PITSLRE protein kinases and Cyclin B1 were immunodetected by the rabbit polyclonal anti PITSLRE antibody (1/1000) (Santa Cruz) and the rabbit polyclonal anti cyclin B1 antibody (dilution 1/1000) (Santa Cruz), respectively. Antibodies were detected with an enhance chemiluminescence kit (Amersham).

Reporter gene assays

For the reporter gene assays, cells were lysed in 25 mM tris phosphate pH 8, 2 mM DTT, 2 mM CDTA, 10 % glycerol, 1% triton X-100.

Firefly luciferase was assayed in a volume of 30 μ l. The reactions were initiated by addition of 15 μ l of luciferase assay/substrate buffer (40 mM Tricine, 2 mM (MgCO₃)₄Mg(OH)₂.H₂O, 5 mM MgSO₄, 66 mM DTT, .2 mM EDTA, .5 mM CoA, 1 mM ATP, 1mM D-luciferin) to 15 μ l cell lysate. The light signal was measured using a Top-Count (Packard).

β -galactosdase was measured in a volume of 200 μ l. 20 μ l lysate was added to 160 μ l substrate buffer (60 mM Na₂HPO₄, 10 mM KCl, 1 mM β -ME) reaction was initiatet by adding 20 μ l of 50 mM chlorophenolred- β -D galactopyranoside (CPRG). The colorimetric signal was measured at 595 nM.

Cellular RNA purification and Northern blotting

Total RNA was isolated making use of the RNAeasy kit (Quiagen) according to manufacturer's instructions.

Total cellular RNA (10 μ g/lane) was denatured in formaldehyde and electrophoresed through a 1.2 % formaldehyde-agarose gel. RNAs were transferred onto a nylon membrane (Amersham) by the capillary blot procedure. The filters were UV cross-linked using a UV Stratalinker apparatus (Stratagene) and were hybridized with the indicated cDNAs labeled with ³²P by randomly primed DNA synthesis. The hybridization probes were Luciferase (a 1700 bp cDNA *NcoI/XbaI* restriction fragment), LacZ (a 800 bp cDNA *Nco I/Clal* restriction fragment), PITSLRE (a 650 bp *PvuII* restriction fragment) and an E-tag probe (5'-ACGCGGTTCCAGCGGATCCGGATACGGCTCCGGCGCACCT-3').

In Vitro Transcription and Translation

Plasmids were linearized downstream from the 3' end of the coding sequence. Capped or uncapped RNAs were generated in vitro by sp6 RNA polymerase according to the manufacturer's instructions (AMBION), with or without adding m7GpppG (final concentration, 3.75 mM) (AMBION) to the reaction mixture, repectively. Transcripts isolated by Lithium Chloride precipitation and quantitated by A260 were used as a template in an vitro translation reaction according to the

manufacturer's instructions (Promega) in the absence or presence of Ba/F3 cell extracts.

For the preparation Ba/F3 cell extracts, Ba/F3 cells were harvested by centrifugation, and washed three times with PBS. The cell pellet was lysed. Nuclei were removed by centrifugation for 5 min, and the mitochondrial fraction was subsequently removed by centrifugation for 20 min at 12000 x g. The supernatant was dialyzed for 2 hours against 100 volumes of 10 mM Hepes, 90 mM potassium acetate, 1.5 mM Mg acetate and 2.5 mM DTT, pH 7.4. The dialysed lysate was again centrifuged for 10 min at 12000 x g, and the supernatant was stored -80 °C for at least 24 hours. The lysate was thawed and centrifuged for 10 min at 12000 x g. The supernatant was treated with micrococcal nuclease (15 µg per mililiter of extract) in the presence of CaCl (7.5 ml of .1M CaCl per mililiter of extract) at 20°C for 15 min. The reaction was terminated by the addition of 200 mM EGTA (15 µl per mililiter of extract). The lysate was adjusted to 10 % glycerol and stored in small portions at -80 °C (Molla et al., 1991).

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Sequence listingSEQ.ID.NO. 1

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 AATCAGAGGAGGAAGAGGAAGAGGAGGAGGAGGAGACCGGCAGCAACTCTGAGGAGGCATCA
 GAGCAGTCTGCCGAAGAAGTAAGTGAGGAAGAAATG

SEQ.ID.NO.2

GACAUUCAGCGACAGCGAGAGGAAGACCAGCUCGGCCGAGUCCUCGUAGCA
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 GAGGAAGGGAGCACCAGUGAAGAAUCAGAGGAGGAAGAGGAAGAGGAGGAG
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SEQ ID.NO.3

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SEQ.ID.NO.4

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SEQ.ID.NO.5

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SEQ.ID.NO.6

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 AATCAGAGGAGGAAGAGGAAGAGGAGGAGGAAGGGAGCAGCAACTCTGAGGAAGGCATCA
 GAGCAGTCTGCCGAAGAAGTAAGTGAGGAAGAAATG

SEQ.ID.NO.1 is the IRES specified sequence according to the invention

SEQ. ID. NO.2 concerns the corresponding RNA sequence of the IRES element

SEQ.ID.NO.3 shows the nucleotide sequence of PITSLRE protein kinase (p110pitslre) (isoform α 2-2). The IRES-activity containing sequence is underlined and corresponds to SEQ.ID.NO.1.

SEQ.ID.NO.4 is the overlapping sequence between SEQ.ID.NO.1 and SEQ.ID.NO.5.

SEQ.ID.NO.5 is a sequence comprising an extension to the left end of SEQ.ID.NO.4.

SEQ.ID.NO.6 is a sequence comprising SEQ.ID.NO.5 and SEQ.ID.NO.1 with the overlapping SEQ.ID.NO.4 included.

Claims

1. A nucleotide sequence enabling a cell cycle dependent initiation of translation of mRNA.
2. A nucleotide sequence according to claim 1 wherein the sequence is an IRES sequence.
3. A nucleotide sequence according to claims 1 or 2 wherein the cell cycle dependency is a G2 cell cycle dependency.
4. An isolated and/or recombinant nucleic acid molecule, preferably DNA, encoding at least a functional part of an eukaryotic internal ribosomal entry site, which site in the mitotic PITSLRE protein kinase gene comprises the sequence as depicted in SEQ.ID.NO. 1 or a functional part thereof.
5. A nucleic acid molecule according to claim 4 comprising at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence at least substantially homologous thereto.
6. A nucleic acid molecule according to claim 4 comprising at least a part of the sequence as depicted in SEQ.ID.NO.1 or a sequence which hybridizes under conventional conditions to at least a part of said sequence or its complementary sequence.
7. An isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 4.
8. An isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 5.
9. An isolated and/or recombinant nucleic acid molecule, preferably DNA, comprising at least the sequence as depicted in SEQ.ID.NO. 6.
10. A chimeric gene comprising the following operably linked polynucleotides:
 - a) a nucleic acid molecule according to any of the claims 1 to 9
 - b) one or more control sequences.

11. A vector comprising at least a nucleic acid molecule according to any of the claims 1 to 9 or comprising a chimeric gene according to claim 10.
12. The vector according to claim 11 wherein the vector is an expression vector containing at least a single promoter.
13. An eukaryotic host cell comprising a nucleic acid molecule according to any of the claims 1 to 9 or comprising a chimeric gene according to claim 10.
14. An expression system comprising an eukaryotic host cell according to claim 13.
15. A method for cap-independent translation of mRNA by including in an expression vector a translation control element or analogues thereof having the nucleic acid molecule as set forth in SEQ.ID.NO.1 and/or SEQ.ID.NO.4.
16. Use of any of the sequences of claims 1 to 9 to induce a cell cycle dependent initiation of translation in eukaryotic cells.
17. Any of the sequences of claims 1 to 9 for use in accordance with claim 16 wherein the sequence is a cell cycle dependent IRES sequence.
18. Use according to claim 17 wherein the cell cycle dependent IRES sequence is a G2-dependent IRES sequence.
19. Use of a vector of claims 11 or 12 or any of the sequences according to claims 1 to 9 for the preparation of a pharmaceutical composition for the treatment and/or prevention of a disease by gene therapy.

Abstract

The current invention relates to two isoforms, p110 and p58 of PITSLRE protein kinase, which can be translated from the same p110 ($\alpha 2\text{-}2$) mRNA by an internal ribosome entry process. This means that p110 and p58, two proteins with putative different functions, are translated from a single mRNA species by using two AUGs within the same reading frame. These two proteins share the 439 C-terminal amino acids that contain the kinase domain. The IRES in the polycistronic p110 mRNA is the first IRES completely localized in the coding region of a cellular mRNA. Moreover, it was unexpectedly found that the IRES element is cell cycle regulated. Translation of p58 occurs in the G2 stage of the cell cycle.

Figure 1: Cell- cycle-dependent expression of p58PITSRE in Ba/F3/p110PITSRE-E-tag transfectants. (A) Western blot of p110PITSRE-E-tag with α E-tag antibody in Ba/F3/p110PITSRE-E-tag transfectants during cell cycle progression. (B) Cell-cycle analysis of DNA content, as measured by propidium iodide staining, of Ba/F3/p110PITSRE-E-tag transfectants released from a G1 block induced by growthfactor depletion.

Figure 2: p58PITSRE is expressed by a mechanism of internal initiation of translation.

(A) Upper: Western blot of p110PITSRE-E-tag with α E-tag antibody in Ba/F3/p110PITSRE-E-tag transfectants during cell cycle progression. The same lysates were also tested for cyclin B expression. Cyclin B is a marker of the G2 stage of the cell-cycle. Lower: Northern blot analysis with an E-tag specific probe.

(B) Western blot analysis of p110ATG mut-E-tag with α E-tag antibody in Ba/F3/p110ATG mut-E-tag transfectants during cell cycle progression.

Figure 3: Strongly enhanced expression of endogenous p58PITSRE kinase in the G2 stage of the cell-cycle. Analysis of endogenous PITSRE kinase expression in Ba/F3 cells during cell cycle progression. The different cell lysates were analysed by Western blot with an α PITSRE kinase antibody (Santa Cruz). This antibody recognizes the C-terminal part of the kinase domain. As a control for the G2 stage, the same lysates were tested for cyclin B expression.

Figure 4: Identification of an IRES in the coding region of PITSRE mRNA. (A) Diagram of a dicystronic mRNA. The coding regions for Luc and LacZ expression are separated by an ICS (Inter Cystronic Sequence) element. (B) Schematic representation of the different PITSRE kinase specific ICSes cloned inbetween the coding regions for Luc and LacZ in the respective dicystronic constructs.

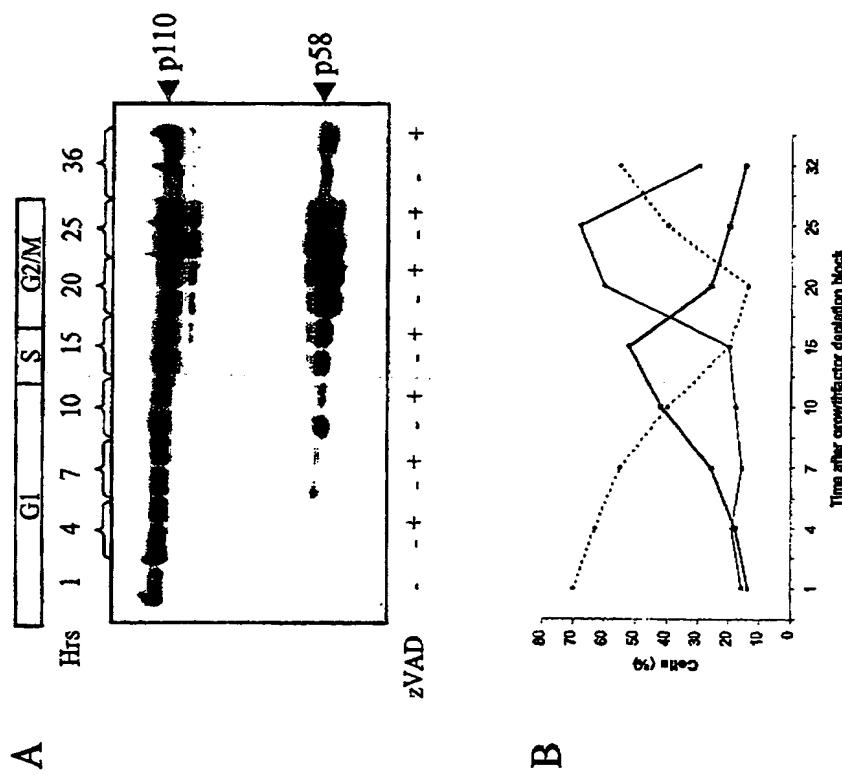
(C) The dicystronic plasmids were transfected in 293T cells. Luciferase and LacZ activity was measured in the respective lysates. The values represent the ratio between the LacZ and the Luc activities. The values represented here are representative for four independent transfection experiments.

Figure 5: Luciferase and LacZ expression from different dicystronic mRNA molecules. (A) Western blot analysis of cell lysates of 293T cells transfected with the indicated dicystronic construct. The upper and the lower panels show detection with α Luc and α LacZ antibody respectively. (B) Comparison of Luciferase and LacZ expression from di1 mRNA and HPdi1 mRNA by Western blot analysis with respectively α Luc and α LacZ antibodies. HPdi1 contains a stable RNA hairpin in the 5'noncoding region of Luc.

Figure 6: The G2 stage of the cell cycle facilitates internal initiation of translation on dicystronic mRNA, di-1. (A) Expression of Luc (panel left) and LacZ (panel right) in asynchronous cell populations of two clones of Ba/F3/di-1 transfecants. (B) Ires activity in different Ba/F3/di-1 transfecants. Cell lysates of Ba/F3/di-1 transfecants synchronized in the G1 (dark bars) or G2 stage (white bars) of the cell cycle were tested for LacZ and Luc activity. To correct for the amount of mRNA we show the ratios of the Luc and the LacZ activities measured in the different clones. The absolute values of Luciferase activity varied in the different clones.

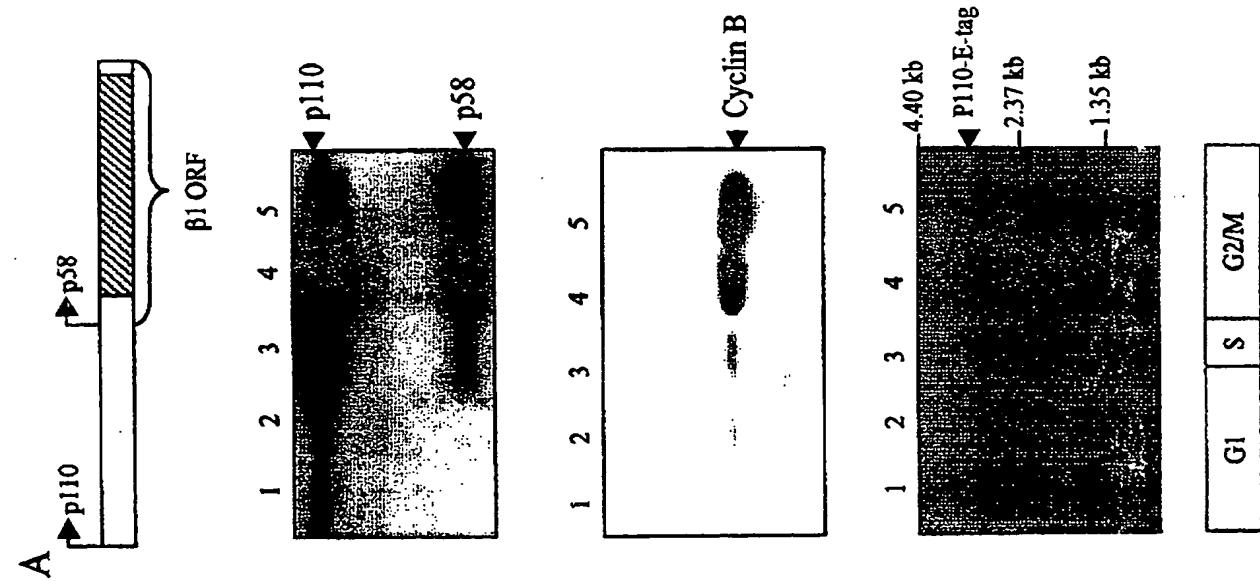
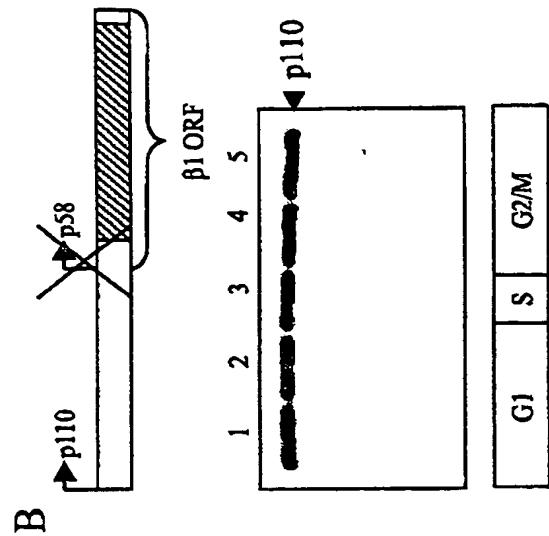
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Figure 1



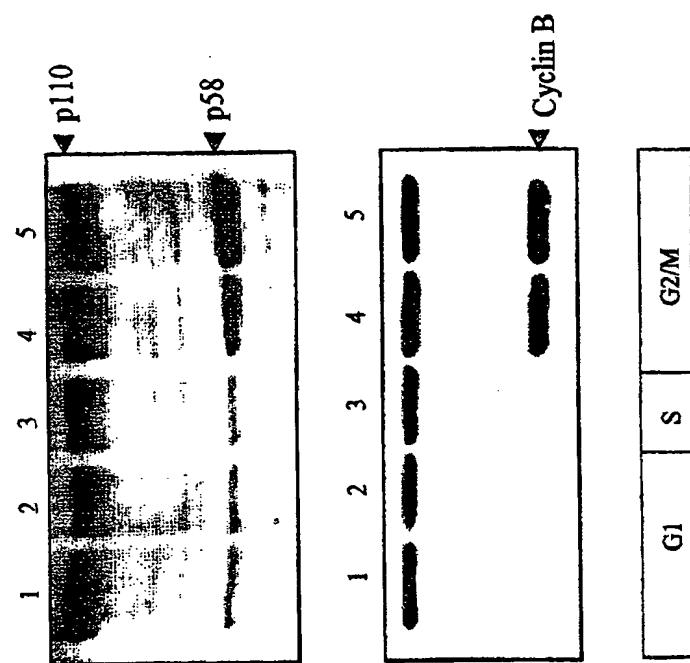
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Figure 2



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Figure 3



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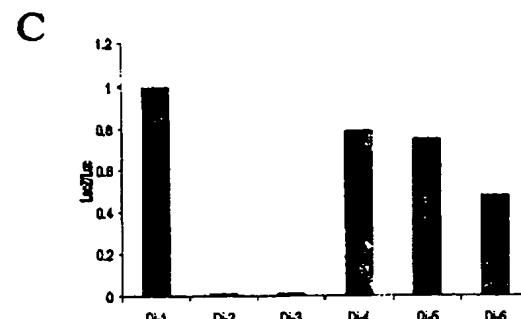
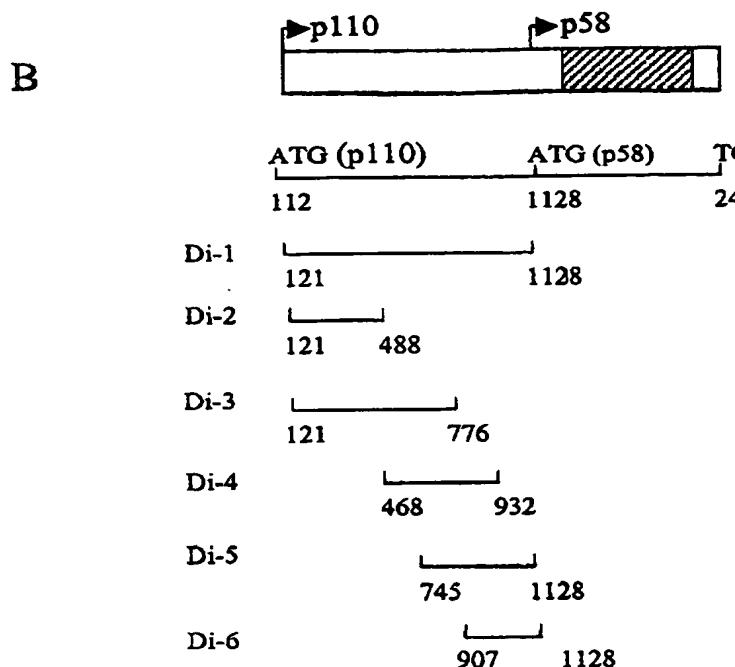
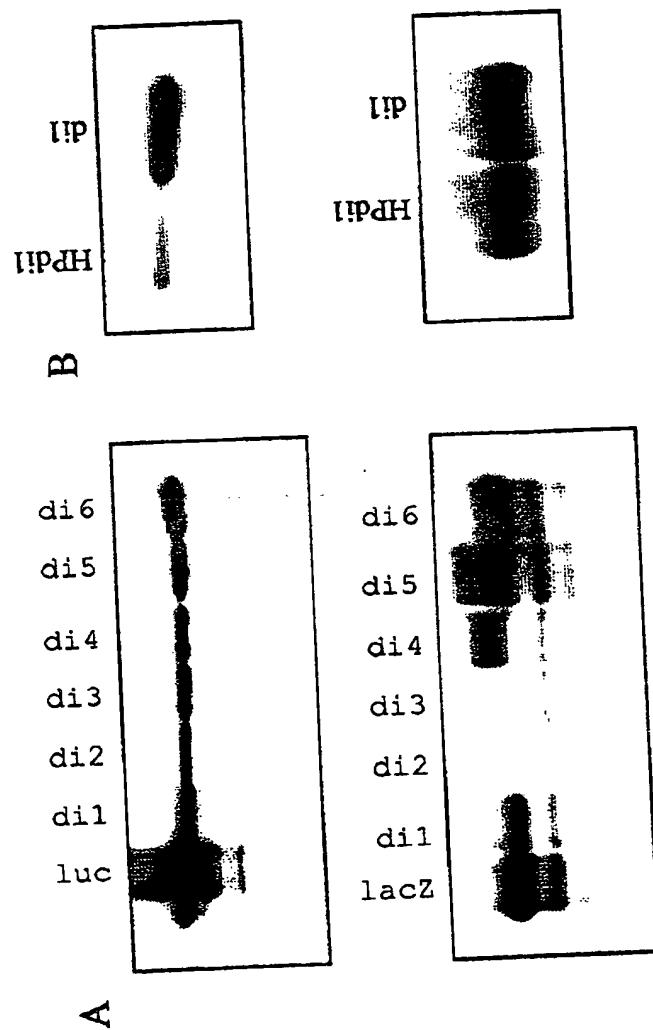


Figure 4

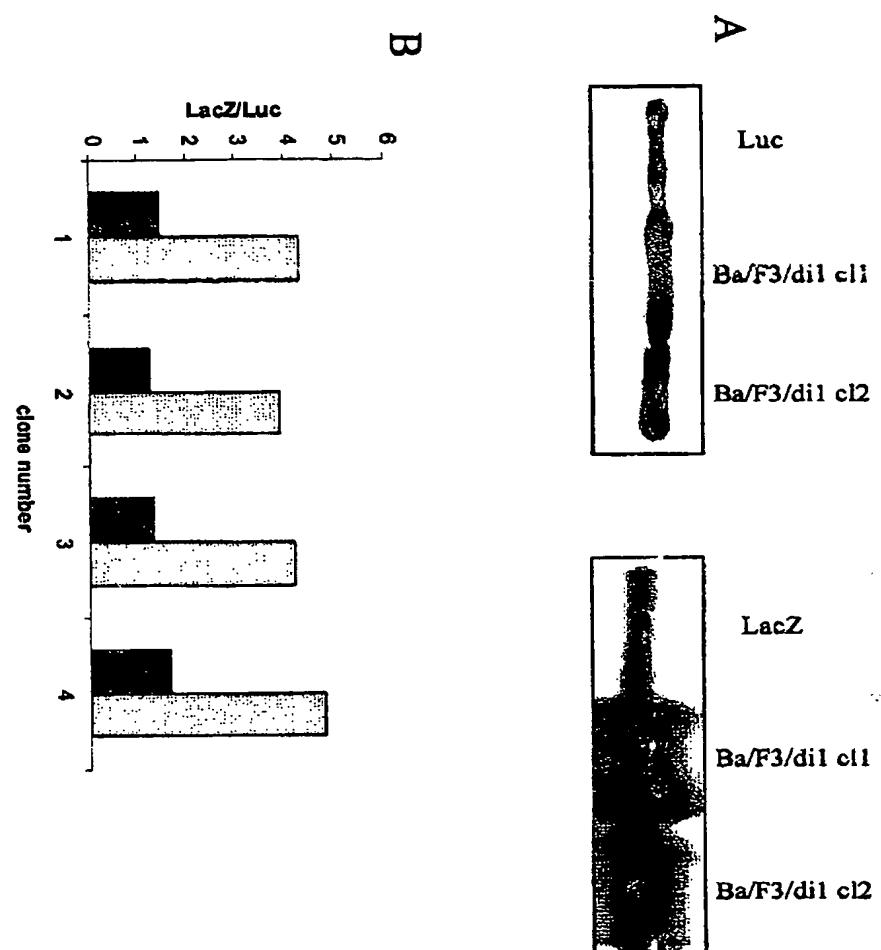
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Figure 5



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Figure 6



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